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Progress in Anthrax Diagnostic by H. Heidenmuller

From the Bavarian Institute for Animal Epidemics in Schleissheim.
Special reprint from the "Monatshefte fur Tierheilkunde" Vol 6 No 8, 1954
Publisher: Ferdinand Enke, Stuttgart With 2 plates and 1 table

In a previous publication (Ref 13) the difficulties of laboratory diagnosis of anthrax were presented and the reliability of the individual diagnostic processes were evaluated. In summing up it was established that in all cases several test methods should be applied in order to make a diagnosis of "anthrax", and that no single property of the anthrax bacillus (perhaps with the exception of its growth in beef broth as flaky sediment in otherwise consistently clear culture medium) is sufficiently specific by itself in order to serve as the exclusive criterion. In the meantime we isolated an anthrax strain which grows in broth only with turbidness. Jensen and Klecmoyer (Ref 4) describe two similar strains in connection with their test of 50 anthrax strains. Standfuss (Ref 11) points to the superiority of the animal test over the culture method: In 256 examinations of dried skin samples from overseas, anthrax could be diagnosed 133 times only through the animal test, 110 times by the animal test and culture, and only 13 times by culture alone. The subcutaneously infected mice died on the 1st-20th day following infection. Since anthrax bacilli could be found in the blood of the heart and in the spleen not sooner than the third day after injection, Standfuss stresses the necessity for an examination of the locale of injection in case the mice should die sooner. According to Martos (Ref 6) , one single anthrax germ killed only 28% of the infected test mice while 20 germs were required to kill every one. However, this data cannot be considered as being always applicable, in view of possible fluctuations in virulence and

changeable susceptibility of the inoculated animals.

Testing by Nordberg (Ref 7) lead to a considerable limitation of the usefulness of the animal test in anthrax diagnostic. In tests of anthrax-like strains 21 of 42 (50%) strains of the type B. mycoides killed mice subcutaneously infected with 0.2-0.5 ccm culture within 20-96 hours after infection. These strains were apathogenic for guinea pigs. Twenty-three of 52 (40%) strains labeled as B. cereus also were able to kill mice after 18-144 hours following infection, and 8 cultures were lethal to guinea pigs within 20-90 hours after subcutaneous dispensation of 0.5-1.0 ccm. Rabbits remained resistant in every case. It was positively determined by means of tests with other methods that anthrax was not involved in any of the tested strains.

The foregoing test results remove the basis of Plan I for revision of export regulations in the Prussian Export Code of the Cattle Epidemics Law (RdErl.d.MdI(KdI) v.1.1.193[illegible]-IIIb 7719/32). There microscopic examination and the stratiform test after Ascoli were prescribed, to be expanded by culture on agar plates as well as inoculation of mice, in case one or both of the former have negative or doubtful results.

Since none of the methods mentioned here can safely lead to the diagnosis of anthrax (Ref 1, 5, 8, 13, 14), Nordberg (Ref 7) suggests the improvement of the usual bacteriologic test methods by the growing of suspicious cultures in inactivated horse serum and in socalled transmigration media. The latter are composed of semi-solid substrates with only a low agar content which can be easily "wandered through" by motile germs, and thus are especially suited to the study of bacterial motility. Nordberg noticed in his test of 96 anthracoid strains from several European countries, South Africa and North

and South America, that 42 strains labeled as B. mycoides as well as 52 strains identified as B. cereus were motile in transmigration media after 6-8 hours of incubation, and on swarm agar plates after at least 6-8 hours, at the latest 24 hours in the incubator. These strains did not grow at any time in inactivated horse serum. Two strains classified as B. cereus were nonmotile in the above mentioned media and showed a good growth after incubation for 4 hours in horse serum, during which a partial encapsulation was also noted. These two strains were able to kill mice, hamsters, guinea pigs and rabbits, and were identified as genuine anthrax strains.

According to Sievers (Ref 10), transmigration nutrients make possible a more objective study of bacterial motility than that which is afforded by the hanging drop. As early as 1900 Gabritschewsky (Ref 3) used such nutrients in the form of joined tubes separated by a cotton tampon. Others (literature in Seidel (Ref 9) used a U-shaped tube, slightly extended in the middle, in which both legs are filled with sea sand and covered with nutrient broth. One side was inoculated, the other side used for withdrawals after a 20-hour incubation. Fischer (Ref 2) worked with transmigration nutrients consisting of semi-solid agar, and based on this Vahlne (Ref 12) created the transgression method by the use of a U-shaped tube with 0.1% agar. According to Seidel (Ref 9) transmigration and transgression nutrients can be successfully utilized in the testing of motility at bacterial meat examinations.

A new method of bacterial differential diagnosis of anthrax was described by Jensen and Kleemeyer (Ref 4). Their "string of pearls test" is based on the fact that viable anthrax bacilli experience a form change in penicillin-containing nutrients, depending on the penicillin concentration. The range of 0.5 to 0.05 I.E./ccm proved to be the most suitable concentration; in such

media the anthrax bacilli consistently assume a globular shape and the anthrax bacillus threads consequently resemble strings of pearls. This form change never occured in anthrax-like bacilli. Of numerous tested substances only penicillin was able to cause this change. During the testing of 50 anthrax strains and 43 anthrax-like strains or ther aerobic sporeformers, neither growth in broth, colony form on agar, the stratiform test after Escoli, pathogenicity tests with mice and guinea pigs, nor motility tests permitted of a positive diagnosis, while the growth characteristic of the anthrax bacillus in the gelatin stab ("reversed fir tree") and the string of pearls test proved to be positively specific. The usefulness of the gelatin stab for differential diagnostic purposes has been refuted, however, from another quarter (Ref 5).

In our own tests the newer diagnostic methods (Ref 4, 7) were to be examined for their usefulness and compared to the traditional test processes.

For this purpose 12 anthrax strains (1 from horses, 1 from mice, 1 from swine, 7 from cattle, 2 of unknown origin) were available to us, as well as 28 anthrax-like bacillus strains, of which 4 were labeled as B. subtilis, 1 as B. cereus, 3 as B. mycoides and 10 as B. mesentericus, while the remaining 10 strains only bore data of origin. All strains were examined for aspects listed in the table. (Page 5)

It is apparent from this summary that in the mice test 1 anthrax strain behaved atypically (pathogenic for mice). Two other strains showed a different growth in broth (with turbidness of the nutrient).

A large number of anthracoid strains showed a true "anthrax-likeness": 17 in respect to their colony form and morphology, 8 in regard to their lack of motion, all strains respecting their staining after Gram, 3 regarding the

TABLE 1

	12 anthrax strains	28 anthrax-like aerobic sporeformers
1. Colony on agar	all spiral nebula	17 spiral nebula 11 unlike anthrax
2. Appearance of bacille (culture)	typical bamboo-like rods	17 anthrax-like 11 unlike anthrax
3. Stain after Gram	all gram positive	all gram positive
4. Motility: 0.5% agar :	nonmotile	20 motile 8 nonmotile
transmigration nutrient :	nonmotile	
5. Hemolysis (blood agar) 24-hr observ.	all negative	25 positive 3 negative
6. Thermo precipitation after ascoli	all positive	11 positive 3 doubtful 14 negative
7. Pathogenicity (21-day observation)		
white mice:	11 pos, 1 negative	2 positive, 26 negative
guinea pigs:	not tested	28 negative
8. Growth in broth (24 hours)	10 with typical flake in otherwise clear broth, 2 with turbid broth	4 with flake in clear broth 24 with turbidness, partly with surface film
9. Growth in inactive horse serum (4 hours)	meagre growth, no encapsulation	7 meagre growth 21 no growth
10. String of pearls test	all positive	all negative
11. Gelatin stab culture (10-day observation)	all typical shape of reversed fir tree, liquefaction of gelatin	3 times shape of reversed fir tree, 8 times a very similar form, 17 times unlike anthrax, 15 times liquefaction of gelatin between 3 and 6 days

missing hemolytic ability and 11 in respect to the thermo precipitation reaction. The fact that the latter is by no means to be taken as an infallible diagnostic is stressed by Prof. Ascoli himself (Ref 8), and has also been described by Zieger (Ref 14). Of the anthrax-like strains 2 more were pathogenic for mice, 4 showed the same growth as anthrax in broth, 7 had a weak growth in horse serum and 3 or 8 showed the same picture (or at least a very similar one) as the genuine anthrax causer in gelatin stab culture. Most anthracoid strains resembled the anthrax bacillus in several properties.

An absolute difference was apparent in the growth on penicillin-containing agar. The "string of pearls test" therefore proved to be positively specific in our tests also, and can be considered a valuable contribution to anthrax diagnostic. The required nutrients can be made simply and inexpensively; they are usable for at least 3 months, and the test results can be established with certainty, depending on the purity of the submitted material, at the earlies between 3 and 18 hours after receipt. This method does not suffer by the fact that an anthrax mutant behaved negatively in the "string of pearls test" (Ref 4). This mutant, isolated by Tomscik, in contrast to its original strain was apathogenic for mice and guinea pigs, atypical morphologically and motile. Thus it had lost several important anthrax characteristics and could hardly any longer be called a cause of anthrax.

In the application of the "string of pearls test" we utilized the technique described by Jensen and Kleemeyer, with due consideration for our own experiences. A broth tube is inoculated from a culture on a solid nutrient (agar, blood agar) and incubated for 3 hours at 37°C. If a microscopic examination of the submitted piece of organ (spleen) indicates an abundance of possible anthrax germs, a tissue sample may be directly incu-

bated in broth in order to gain time. After 3 hours the broth culture, which as arule shows a distinct growth, is thoroughly shaken and one loop each is applied to the prepared nutrients. For the preparation of penicillin media the measured amount of penicillin solution is added to the usual infuso decoction (1 part meat to 2 parts water) cooled to 45-50°C in such a manner that nutrients of 0.5 I.E./ccm and 0.05 I.E./ccm result. In this connection it has proved to be practical to draw penicillin in as small amounts as possible (ampulla of 5,000 I.E.), since only a fraction is used for the nutrients. The prepared medium may not get too soft by the addition of penicillin. After solidification the penicillin agar should not be thicker than 2 mm. For the test itself one wafer apiece of both penicillin nutrients (cut out with an agglutination tube) are applied to a slide which has previously been subjected to a flame, in addition to one ordinary wafer of 2% agar. The latter serves as growth control. Following inoculation of the three media wafers the slide is incubated in a moist chamber (petri dish) for 2-3 hours, and subsequently examined under lens magnification or a weak dry system. Examination by means of a cover glass and oil immersion does not offer any advantages. If anthrax is involved the bacilli on the agar without penicillin content have grown to long sinuous threads (Fig 1). On the agar with a 0.5 I.E./ccm penicillin content the growth is strongly impeded; the bacilli are partly distended, while on the third nutrient (0.05 I.E./ccm penicillin content) the bacilli appear (under suitable illumination) as glowing clusters of grapes or strings of pearls (Fig 2). Anthrax-like organisms on all 3 media always show a uniform growth without change in form.

The penicillin nutrients proved to be fully usable 14 weeks after their preparation; the impediment to bacillus growth of the more highly concentrated

penicillin agar merely was lessened due to the weakening of the effects of penicillin; and here too the organisms grew in pretty strings of pearls. Transmigration media after Nordberg (Ref 7) are unnecessary if 0.5% agar is used for determination of the motility of bacilli. In this respect too we successfully used slides which were covered with a nutrient wafer (cut out with an agglutination tube) and incubated in a moist chamber after inoculation with a trace of solid culture. In the case of motile cultures the nutrient was covered with a fine, turbid layer of swarming bacilli; negative motility of the germs was indicated by culture growth; the bare nutrient, however, retained a shiny surface.

Horse serum culture (Ref 7) proved to be less reliable. It is unnecessary when the "string of pearls test" is utilized. Encapsulation of anthrax in horse serum, as sometimes noted by Nordberg (Ref 7), could not be confirmed by us in any case (Giemsa and methylene blue stains).

SUMMARY

Twelve anthrax and 28 anthrax-like aerobic strains were comparatively tested. In connection with 11 test processes the "string of pearls test" alone proved to be positively specific. This procedure makes possible a speedy and certain diagnosis of anthrax; therefore it is suggested that this method be established in anthrax diagnostic.

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EXPLANATION OF PLATES

Fig. 1. Anthrax agar culture of 3 hours. Bacilli are arranged in long threads, partly in the form of skeins (180X).

Fig. 2. Anthrax culture on agar with 0.05 I.E. penicillin/ccm after 3 hours. Due to the influence of penicillin the bacilli have changed to more or less rotund objects, lieing in the form of strings of pearls. (360 X)

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